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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Chemokines play a pivotal role in the maturation of the immune system, and in the initiation, and maintenance of an immune response. Because of their key role in the immune response, the aberrant expression of chemokines can have a profound effect on the ability of T cells to respond to antigen. We have found that several breast cancer cell lines produced chemokines capable of recruiting T cells. However, instead of increasing anti-tumor immunity, the tumor-derived chemokines may have prevented an effective immune response by desensitizing T-cell chemokine receptors. Our hypothesis is that disrupting the synthesis of tumor-derived chemokines (using anti-sense technology) will remove tumor-induced immune suppression and enhance the immunogenicity of the tumor. In order to test this hypothesis we generated stable clones that lack CCL2 and CCL5 production compared to the parental tumor cell line. Using these tumors we found that both CCL2 and CCL5 impair the T cell response to the murine mammary carcinoma 4T1. Moreover, tumor-derived CCL5 impaired T cell chemotactic activity and enhanced growth of the tumor in vivo. In addition, we found that tumor-derived CXCL1 acts as an autocrine growth factor and angiogenesis factor for 4T1.

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Chemokine, receptor desensitization, adaptive immunity, immune suppression

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INTRODUCTION

Chemokines play a pivotal role in the maturation of the immune system, and in the initiation, and maintenance of an immune response [1]. Because of their key role in the immune response, aberrant expression of chemokines can have a profound effect on the ability of T cells to respond to antigen. We have found that several breast cancer cell lines produced chemokines [Regulated upon activation, normal T cell expressed and secreted (RANTES, CCL5) and monocyte chemotactic factor-1 (MCP-1, CCL2)] capable of recruiting T cells, as well as the chemokine KC (CXCL1) [2]. Additionally, supernatants derived from the tumor cell line 4T1 could mediate the chemotaxis of T cells. However, instead of increasing anti-tumor immunity, the tumor-derived chemokines may have prevented an effective immune response by desensitizing T-cell chemokine receptors [2]. The receptors for CCL5 and CCL2 on T cells were desensitized in tumor-bearing animals. Moreover, there was cross-receptor desensitization of the CC chemokine receptor 7 (CCR7), which impaired the ability of the T cells to respond to secondary lymphoid chemokine (SLC, CCL21). These data indicate that the aberrant expression of tumor-derived chemokines may help tumors escape immune attack. Our hypothesis is that disrupting synthesis of tumor-derived chemokines (using anti-sense technology) will remove tumor-induced immune suppression and enhance the immunogenicity of the tumor. We will determine whether the T cells are better able to elicit an anti-tumor immune response by comparing the immunogenicity of the tumors that do and do not express chemokines. These tumor cells will be evaluated by immunization/challenge experiments and by the ability to generate tumor-specific T cells in tumor draining lymph nodes.

BODY

The first objective of the project was to generate sense and anti-sense constructs capable of inhibiting synthesis of the tumor-derived chemokines CCL5, CCL2, and CXCL1. This objective has been completed. For the second and third objectives, transfection of the 4T1-9 tumor cell line has been accomplished with the sense and anti-sense CCL5, CCL2 and CXCL1 eukaryotic expression vectors. Progress with each chemokine is described separately below.

Statement of Work

Objective 1. Generate anti-sense constructs capable of inhibiting synthesis of tumor-derived chemokines, months 1-9:
a. PCR amplify CCL5, CCL2 and CXCL1 from the murine breast cancer cell line 4T1.
b. Ligate the chemokines into the T-vector.
c. Transform the vector containing the chemokines into competent E. coli.
d. Screen for clones that contain the correct insert by blue/white screening and a BamH1/Not1 restriction digest.
e. Digest the correct clones with BamH1 and Not1 and gel purify the chemokine DNA.
f. Separately ligate each chemokine into the vector in the reverse orientation.
milestone #1-Single antisense vectors constructed
g. Ligate together the CCL5, CCL2, and CXCL1 PCR products.
h. Transform the vector containing all three chemokines into competent E. coli.
i. Screen for the clone that contains the correct insert by blue/white screening and a BamH1/Not1 restriction digest.
j. Digest the correct clone with BamH1 and Not1 and gel purify the chemokine DNA.
k. Ligate the chemokine DNA into the vector in the reverse orientation for the triple antisense construct.
milestone #2-Triple antisense vector constructed

*Objective 2.*Transduce and clone tumor cells that lack production of chemokines, months 10-18:
a. Package the retroviral construct by transfecting the PA317 packing cell line.
b. Collect, concentrate and titre the virions.
c. Transduce 4T1 with each construct.
d. Drug selection and reclone the tumor cells.
e. Screen the transduced clones by RT-PCR for the presence of the antisense construct, and chemokine mRNA synthesis.
f. Screen the transduced clones by ELISA for chemokine protein synthesis.
g. Screen the supernatants from the transduced clones for T cell chemotactic ability.
milestone #3-Tumor cells cloned with antisense transgenes.

Objective 3. Examine the ability of tumor cells lacking chemokines to induce chemokine receptor desensitization and for increased immunogenicity, months 19-36:
a. Compare desensitization of chemokine receptors on T cells from 4T1 tumor- bearing mice to T cells from the transduced 4T1 tumor-bearing mice. We will perform this assay three times with each of the anti-sense expressing clones.
b. Compare the immunogenicity of the clones by immunization/challenge experiments.
c. Compare the immunogenicity of the clones by determining their ability to generate tumor-specific T cells in vaccine draining lymph nodes.
milestone #4-Evaluate whether targeted disruption of tumor-derived chemokine synthesis reverses tumor-induced immune suppression.

Impact of tumor-derived CCL5

CCL5 is constitutively expressed by the 4T1 tumor cell line as well as T cells, epithelial cells and platelets following exposure to inflammatory agents or mitogens [1, 2]. Mast cells, T cells, natural killer cells, dendritic cells, eosinophils and basophils are capable of responding to CCL5 via CCR1, CCR3, and/or CCR5 [3]. In order to study the impact of tumor-derived CCL5 on anti-tumor immunity we attempted to inhibit CCL5 production using anti-sense technology. For this purpose tumor cells were transfected with the sense and anti-sense vectors, cloned and screened for CCL5 production by RT-PCR and ELISA. Sense (R1) and anti-sense (RA5) clones were selected and studies assessing the role of CCL5 in tumor-induced immune suppression have been completed and published (reprint included in appendix) [4]. Collectively, we found that tumor-derived CCL5 enhanced the *in vivo*, but not the *in vitro* growth rate of the tumor cells, impaired T cell chemotactic activity in tumor bearing mice and impaired the ability to generate a tumor-specific T cell response [4]. Finally, using vaccination/challenge experiments we found that, despite the enhanced T cell response, reduction in tumor-derived CCL5 did not improve the immunogenicity of the tumor cells. These experiments completed the CCL5 portion of the project. A separate study utilizing the R1 and RA5 lines generated for this project, revealed that tumor-derived CCL5 enhanced the metastatic ability of the tumor cells. These data were recently accepted for publication [5].

Summary of findings with CCL5

1. The anti-sense strategy can effectively block tumor-derived CCL5 production.
2. Tumor-derived CCL5 increases the *in vivo* growth rate of the 4T1 murine mammary carcinoma.
3. Tumor-derived CCL5 inhibits the T cell response to the 4T1 murine mammary carcinoma.
4. Tumor-derived CCL5 alters T cell chemotactic ability in tumor-bearing mice.
5. Tumor-derived CCL5 impairs TIL recruitment to the tumor.
6. Tumor-derived CCL5 did not influence the viability of the T cells or induce a Th1-Th2 switch.
7. Tumor-derived CCL5 did not influence the immunogenicity of the tumor.
8. CCL5 enhances breast cancer metastasis.

Impact of tumor-derived CCL2

CCL2 is constitutively expressed by the 4T1 tumor cell line as well as macrophages, neutrophils, mast cells and fibroblasts during an inflammatory response [1, 2]. Macrophages, dendritic cells and T cells are capable of responding to CCL2 via CCR2 and/or CCR4 [3]. In order to study the impact of tumor-derived CCL2 we attempted to inhibit CCL2 production using an anti-sense eukaryotic expression vector. For this purpose 4T1 tumor cells were transfected and we identified a clone (G7) that did not produce detectable levels (assay sensitivity <15pg/ml) of CCL2. The sense (A4) and anti-sense (G7) expressing clones were expanded and used to determine whether tumor-derived CCL2 influenced the T cell response to cancer. The studies revealed that tumor-derived CCL2 impaired the T cell response, but not the chemotactic ability or immunogenicity of the tumor cells (reprint included in appendix) [6]. These experiments completed the CCL2 portion of the project. A separate study utilizing the A4 and G7 lines generated for this project, revealed that tumor-derived CCL2 has no effect on macrophage effector function. These data were recently accepted for publication [7].

Summary of findings with CCL2

1. The anti-sense strategy can effectively block tumor-derived CCL2 production.
2. Tumor-derived CCL2 does not alter the growth kinetics of the 4T1 murine mammary carcinoma *in vitro* or *in vivo*.
3. Tumor-derived CCL2 decreases the ability of T cells to produce tumor-specific IFN-γ.
4. Recombinant CCL2 can directly down-modulate the ability of T cells to produce IFN-γ.
5. Tumor-derived CCL2 is not responsible for the altered chemotactic ability of T cells in tumor-bearing mice.
6. Modulation of tumor-derived CCL2 did not alter the immunogenicity of the 4T1 murine mammary carcinoma.
7. Tumor-derived CCL2 does not influence macrophage effector function.

Impact of tumor-derived CXCL1

CXCL1 is constitutively expressed by the 4T1 tumor cell line as well as neutrophils, epithelial cells and platelets following exposure to inflammatory agents or mitogens [1, 2]. Mast cells, neutrophils, eosinophils and basophils are capable of responding to CXCL1 via CXCR2, and to a lesser extent CXCR1 [3]. In order to study the impact of tumor-derived CXCL1 we attempted to inhibit CXCL1 production using anti-sense technology. For this purpose the tumor cells were transfected with sense and anti-sense eukaryotic expression vectors, cloned and screened for CXCL1 production by RT-PCR and ELISA. Using two different expression vectors we transfected and screened hundreds of clones and found many that had no or low levels of CXCL1 expression (figure 1A). However, CXCL1 expression could not be suppressed in a stable manner. All clones regained CXCL1 expression (figure 1B). Of significant interest we observed that the clones that initially expressed the lowest levels of CXCL1 grew the slowest in culture, and over time the growth rate increased as well as CXCL1 expression. Thus, it appeared that CXCL1 production was associated with cell growth rate. Previously, it has been reported that the human CXCL1 equivalent (Gro) acts as an autocrine growth factor for human melanoma [8-10]. Therefore, we began to investigate whether CXCL1 functioned as an autocrine growth factor for the murine mammary carcinoma 4T1. If CXCL1 were an autocrine growth factor it would explain the difficulty in generating a CXCL1 negative tumor cell line. For this purpose we assessed whether receptors for CXCL1 were present on the tumor cells. We designed primers for CXCR1 and CXCR2 and found that 4T1 expresses both receptors (figure 2). Next, we evaluated whether neutralization of CXCL1 using a monoclonal antibody could slow the growth of the tumor cells in vitro. Preliminary studies showed that neutralization of CXCL1 could modulate tumor growth in a dose dependent manner (figure 3). These data could explain why we could not generate a stable clone that did not express CXCL1. However, that data was generated with 4T1-9, a subclone of 4T1, and similar studies using the parental 4T1 line revealed that neutralization of CXCL1 did not influence growth of the tumor cells (figure 4), although pertussis toxin could modestly inhibit growth (figure 5). This pertussis toxin induced decrease in growth was not due to pertussis toxin induced toxicity since the cells were 95% viable. As a result, the 4T1-9 line uses CXCL1 as an autocrine growth factor, and the parental 4T1 line has an

autocrine growth factor that can be inhibited upon blocking G protein coupled receptor signaling with pertussis toxin, but it is not CXCL1. Moreover, we found that blockade of CXCL1 inhibited vessel formation using an in vitro angiogenesis assay (table 1). Supernatants from 4T1 (that contained CXCL1) stimulated vessel formation at levels comparable to the positive control (table 1). Whereas neutralization of CXCL1 blocked vessel formation. Collectively, these data indicate that CXCL1 is necessary for growth of the parental breast cancer cell line 4T1 in vivo, and CXCL1 is an autocrine growth factor for 4T1-9. Finally, since CXCL1 was necessary for growth of 4T1 we used a different strategy to inhibit all three chemokines. Using neutralizing antibodies specific for all three chemokines we found that inhibition of CCL2 and CXCL1 or CCL2 and CCL5 inhibited growth of the parental 4T1 line (figure 6). We anticipate submitting the CXCL1 data for publication this fall.

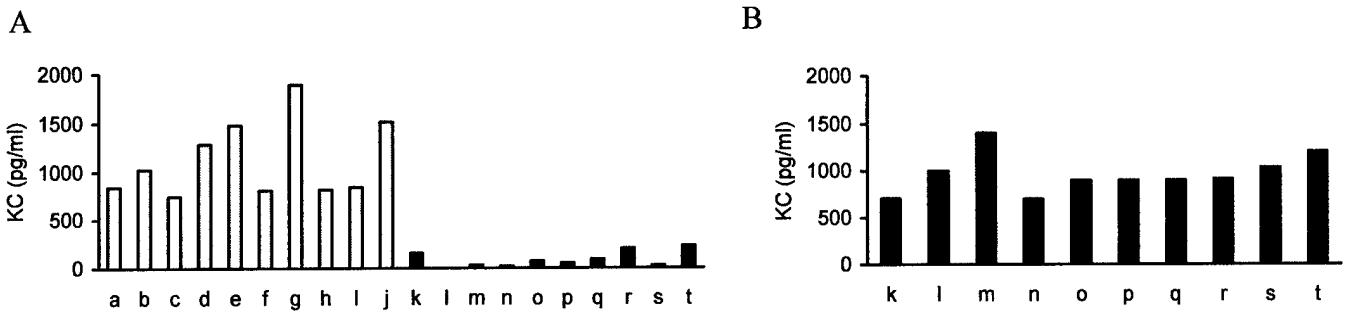

Figure 1. Screening of anti-sense transfected 4T1. A. More than 300 anti-sense transfected clones were screened by ELISA. Lanes a-j represents the vast majority of the clones that were screened. Lanes k-t represents clones that exhibited a decrease in CXCL1 expression. B. When the clones with low levels of CXCL1 expression were grown for another week in culture the level of CXCL1 expression was significantly increased.

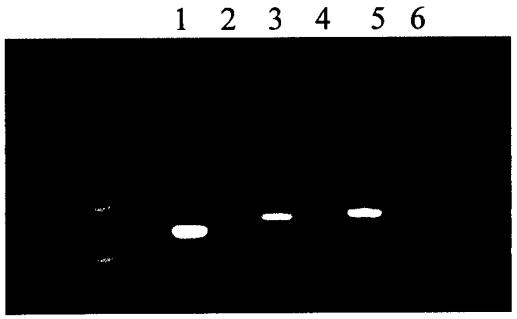

Figure 2. CXCL1 receptor expression. RT-PCR was used to determine whether the 4T1 cells expressed mRNA encoding the CXCL1 receptors; CXCR1 and CXCR2. Lanes 1, 3 and 5 represent GAPDH, CXCR1 and CXCR2 expression respectively. Lanes 2, 4 and 6 are the negative controls. The data are representative of three separate experiments.

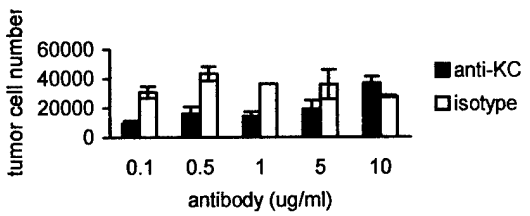

Figure 3. Antibody mediated neutralization of CXCL1. A neutralizing antibody (R&D Systems) specific for murine CXCL1 was added at the indicated concentrations in the presence of the 4T1-9 cells. After 72 hours of culture the tumor cells were harvested and counted. The data are representative of three separate experiments.

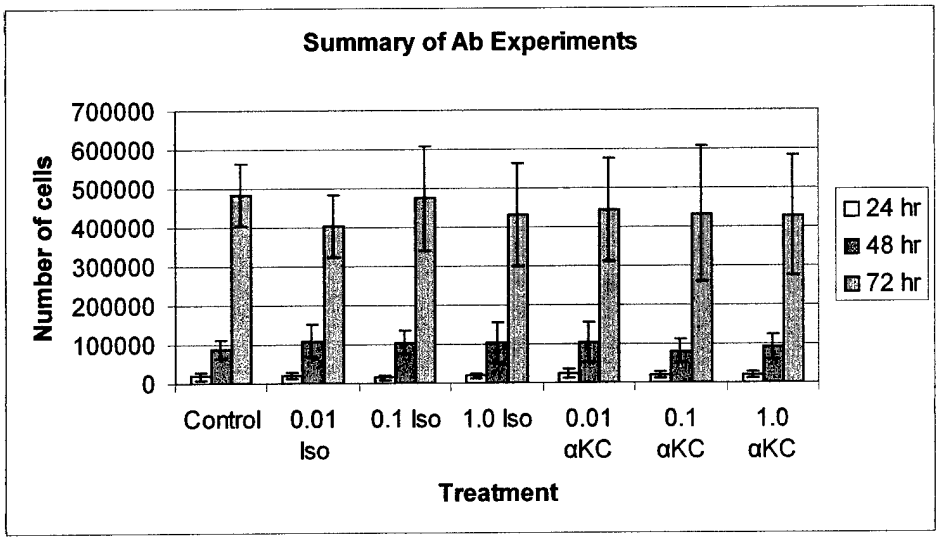

Figure 4. Neutralization of CXCL1 does not inhibit growth of 4T1. 4T1 cells were cultured in the presence of a CXCL1-specific neutralizing antibody (αKC) or an isotype (Iso) control at the indicated concentrations (ug/ml) for 24-72 hours. The cells were recovered at the indicated times by trypsinization and counted. The experiment was repeated three times with similar results.

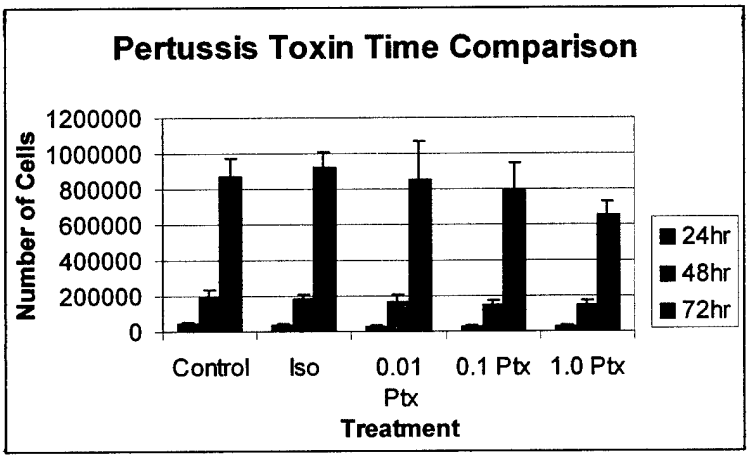

Figure 5. Pertussis toxin modestly inhibits growth of 4T1. 4T1 cells were cultured in the presence of pertussis toxin (Ptx) at the indicated concentrations (ug/ml) for 24-72 hours. The cells were recovered at the indicated times by trypsinization and counted. The experiment was repeated three times with similar results.

Table 1. Vessel Formation.

	positive control	negative control	4T1 supernatants	4T1 supernatants + anti-CXCL1 antibody
vessel formation[a]	+++[b]	0[c]	+++	0

[a]Vessel formation was assessed using HUV-EC (human umbilical cord endothelial cells) and an in vitro angiogenesis assay (Chemicon International).

[b]+++ indicates that clear vessel formation was evident.

[c]0 indicates that no vessel formation was evident.

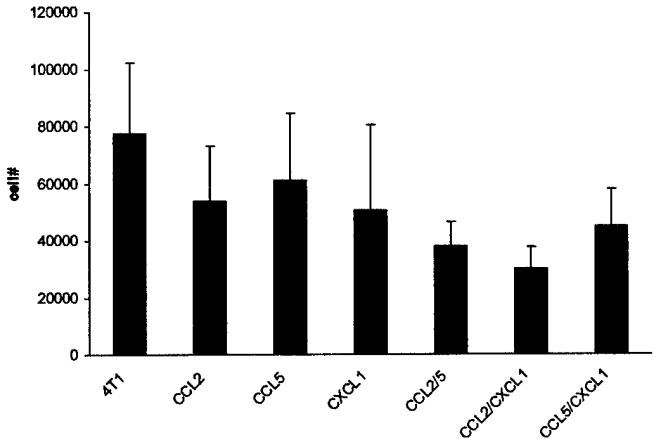

Figure 6. Inhibition of multiple chemokines. 4T1 tumor cells were placed in culture in the presence of the indicated neutralizing antibodies (0.1 ug/ml). Twenty-four hours later the cells were harvested and counted. These data are representative of three separate experiments.

Summary of findings with CXCL1

1. Tumor-derived CXCL1 can be blocked using anti-sense strategy, but the cells regain CXCL1 expression.
2. 4T1 murine mammary carcinoma cells express mRNA encoding the CXCL1 receptors.
3. Neutralization of CXCL1, using a specific antibody, can slow the growth of a subclone of 4T1; 4T1-9.
4. Neutralization of CXCL1 cannot slow the growth of 4T1.
5. Neutralization of CXCL1 and CCL2 inhibits growth of 4T1.
6. Tumor-derived CXCL1 appears to be an angiogenic factor for 4T1.

KEY RESEARCH ACCOMPLISHMENTS

- Cloning of CXCL1, CCL2 and CCL5 gene fragments into a T vector.
- Construction of sense and anti-sense expression vectors for CXCL1, CCL2 and CCL5.
- Generation of stable sense (R1) and anti-sense (RA5) CCL5 expressing tumor cells.
- CCL5 portion of project complete and manuscript published [4]. We have confirmed that CCL5 modulates T cell mediated anti-tumor immunity.
- Generation of stable sense (A4) and anti-sense (G7) CCL2 expressing tumor cells.
- CCL2 portion of project complete and manuscript published [6]. We have confirmed that CCL2 modulates T cell mediated anti-tumor immunity.
- Data generated that indicate CXCL1 participates in growth of the 4T1 tumor cells in vitro and in vivo.

REPORTABLE OUTCOMES

I. Abstracts from meeting presentations. Results from this project were presented at several meetings.

1. 78th annual Pennsylvania Academy of Science (PAS) meeting. title: Blockade of the tumor-derived CCL5 and the impact on T-cell migration. E Adler and RA Kurt. April 2002.
2. Department of Defense Era of Hope meeting. title: Inhibition of tumor-derived CCL2 and anti-tumor immunity. RA Kurt, E Allison, M Shainheit, P Vitiello. Sept 2002.
3. 79th annual PAS meeting. title: Investigating the role of tumor-derived CXCL1 in murine breast cancer. R Harris and RA Kurt. April 2003.
4. American Association of Immunologists meeting. title: Enhancement of anti-tumor immunity by inhibition of tumor-derived CCL5. RA Kurt, E Adler, N Katchen. May 2003.
5. 80th annual PAS meeting. title: Elucidating the role of CXCL1 in murine breast cancer. R Harris and RA Kurt. March 2004.
6. 80th annual PAS meeting. title: Enhancement of anti-tumor immunity by inhibition of tumor-derived CCL2 and CCL5. J Lepre and RA Kurt. March 2004.

II. Grants submitted. Results from this project were used as data for several grants submitted.

1. A grant was submitted to the Komen Foundation using data generated from this project. title: Blockade of chemokine receptor signaling and reversal of tumor-induced immune suppression. October 2002
2. Data generated from this project was submitted to the American Cancer Society for funding. title: Tumor associated chemokines induce desensitization of the T cell receptor. Submitted. October 2002
3. A grant was submitted to The Susan G. Komen Breast Cancer Foundation using data generated from this project. title: Blockade of chemokine receptor signaling and reversal of tumor-induced immune suppression. October 2003
4. An NSF career award has been submitted using data generated from this project. title: Career: integration of research into the undergraduate curriculum. July 2004

III. List of personnel receiving pay from the research effort:
PI: Robert A. Kurt
student researchers: Evan Adler, Peter Vitiello, Mark Brault, Matthew Rausch, Charles Lemken, James Lepre.
List of personnel who worked on the research without receiving pay:
student researchers: Erin Allison, Mara Shainheit, Rachel Harris, Nicholas Katchen

IV. Publications resulting from this project:
1. A dual role for tumor-derived RANTES (CCL5). EP Adler, CA Lemken, NS Katchen, RA Kurt. Immunol. Lett., 90:187-194, 2003.
2. Impact of tumor-derived CCL2 on T cell effector function. PF Vitiello, MG Shainheit, EM Allison, EP Adler, RA Kurt. Immunol. Lett., 91:239-245, 2004.

   Since the cell lines generated for this project were used for other projects we acknowledged the DOD for partial support of that work which was recently accepted for publication.
3. Inhibition of metastasis by down-regulation of tumor-derived CCL5. KA Stormes, CA Lemken JV Lepre, MN Marinucci, RA Kurt. (in press: Breast Cancer Research and Treatment)
4. Impact of tumor-derived CCL2 on macrophage effector function. MS Brault and RA Kurt. (in press: Journal of Biomedicine and Biotechnology)

CONCLUSIONS

We have hypothesized that the constitutive expression of chemokines can impair anti-tumor immunity in a breast cancer model. Using anti-sense technology we were able block CCL5 and CCL2 expression from a murine breast cancer cell line (4T1) that normally constitutively produces these chemokines. We have found that CCL5 and CCL2 inhibited the T cell response to the tumor. Tumor derived CCL5 appeared much more detrimental than CCL2. CCL5 impaired the T cell response, chemotactic activity and enhanced growth and metastasis of the 4T1 murine mammary carcinoma. The generation of 4T1 that lacks CXCL1 expression has proven more difficult. However, the difficulty posed has led to some exciting evidence that CXCL1 acts as an autocrine growth factor and angiogenesis factor for 4T1. Collectively, these data indicate that tumor derived chemokines can suppress the T cell response, inhibit chemotactic activity, enhance metastasis, growth and angiogenesis.

Thus, the data generated with this DOD grant has provided evidence that chemokines expressed by breast cancer can be detrimental to the host. The importance of these findings cannot be overemphasized. Since many specimens from patients with breast cancer express CCL5 and CCL2, these data indicate that inhibition of these chemokines would be an extremely valuable avenue to pursue to benefit patients with breast cancer.

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Appendix

Reprints:

1. A Dual Role for Tumor-Derived RANTES (CCL5). EP Adler, CA Lemken, NS Katchen, and RA Kurt. Immunol. Lett., 90:187-194, 2003.
2. Impact of Tumor-Derived CCL2 on T Cell Effector Function. PF Vitiello, MG Shainheit, EM Allison, EP Adler, and RA Kurt. Immunol. Lett., 91:239-245, 2004.

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A dual role for tumor-derived chemokine RANTES (CCL5)

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Abstract

To investigate the role of tumor-derived CCL5 (regulated upon activation, normal T cell expressed and secreted, RANTES) in tumor immunity we compared the T cell response to tumors derived from the 4T1 murine mammary carcinoma cell line that express different levels of CCL5. Tumors that expressed low levels of CCL5 exhibited a decrease in the in vivo, but not the in vitro, growth rate. In conjunction with the decreased growth rate the tumors that produced lower levels of CCL5 contained a greater number of tumor infiltrating lymphocytes compared to tumors that express normal levels of CCL5. One explanation for these findings was that a reduction in tumor-derived CCL5 prevented the tumor-associated alteration in T cell chemotactic activity. Tumors expressing lower levels of CCL5 also elicited a greater tumor-specific T cell response as evident by examination of recently activated T cells from tumor-draining lymph nodes. However, despite the enhanced T cell response, tumors expressing low levels of CCL5 still grew slower than tumors expressing normal levels of CCL5 in SCID mice. These data are consistent with the ability of CCL5 to upregulate transcription of matrix metalloproteinase-9 (MMP9), which can contribute to angiogenesis and thus, foster growth in vivo. Consequently, these data indicate that tumor-derived CCL5 can inhibit the T cell response and enhance the in vivo growth of murine mammary carcinoma.

Keywords: Tumor immunity; RANTES; CCL5; 4T1; MMP9

1. Introduction

Tumor-derived CCL5 has been detected in many clinical specimens. Niwa et al. [1] examined 43 breast cancer and 23 cervical cancer specimens for CCL5 expression. They reported that plasma levels of CCL5 were greater in patients with progressive disease compared to those in clinical remission [1]. Moreover, plasma levels correlated with disease stage with more advanced stages correlating with higher levels of CCL5 expression [1]. Luboshits et al. [2] examined breast cancer cell lines and sections from breast cancer specimens and reported that the cell lines T47D and MCF-7 as well as 74% of the sections exhibited CCL5 expression. In addition, the levels of CCL5 expression were greater than that found in normal epithelial cells, ductal epithelial cells and benign sections. A subsequent study by the same group suggested that the expression of matrix metalloproteinase-9 (MMP9) may be regulated by CCL5, and therefore, CCL5 may play a role in invasion and metastases [3]. Why this chemokine, which is capable of recruiting T cells, did not increase the immunogenicity of these tumors remains unanswered. It is interesting to point out that breast cancer patients in particular may be especially affected by tumor-derived CCL5 because the gene encoding CCL5 is located on the long arm of chromosome 17, the same area Her2/neu is encoded and an area that is amplified in 30% of patients with breast cancer [4,5]. In addition to cervical and breast cancer, CCL5 expression was reported in four of the eight melanoma lines examined by Mrowietz et al. [6]. Preliminary studies in nude mice suggested a possible role for CCL5 in fostering growth of the melanoma lines.

In this study, we were interested in determining how tumor-derived CCL5 influenced the T cell response to the 4T1 murine mammary carcinoma. 4T1 is a relatively weakly immunogenic tumor cell line that constitutively expresses CCL5 [7]. Previously we reported that mice bearing the 4T1 tumor possess splenic T cells with altered chemokine receptor function [7]. Here, we wanted to know whether inhibiting the tumor cells from producing CCL5 would influence the T cell response to the tumor. In order to inhibit tumor-derived CCL5 we constructed an anti-sense CCL5 eukaryotic expression vector. The tumors generated with this construct

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were used to evaluate the role of tumor-derived CCL5 in anti-tumor immunity. We compared T cell responses elicited by tumors that exhibited a reduction in CCL5 (RA5) to tumors that expressed normal levels of CCL5 (R1, vector control). Analysis revealed that down-regulation of CCL5 could increase T cell effector function, and enhance the localization of tumor-infiltrating lymphocytes. Yet, despite the enhanced T cell response evident upon down-regulation of tumor-derived CCL5, delayed growth of the RA5 tumors in SCID mice suggested that another mechanism was at least partially responsible for the decreased growth rate in vivo. The decreased growth rate evident by the tumors expressing lower levels of CCL5 was associated with a reduction in transcription of the angiogenesis factor MMP9 [8]. Consequently, these data indicate that tumor-derived CCL5 impaired the T cell response and enhanced the in vivo growth of the murine mammary carcinoma.

2. Materials and methods

2.1. Mice and tumor cell lines

For these studies 6–8-week-old female BALB/c mice were used. The BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Food and water were provided ad libitum. SCID mice were purchased from Harlan Scientific (Indianapolis, IN). All of the tumor cells used in this study (4T1, 4T1-9, R1, RA5, and SM1) were maintained in complete RPMI (cRPMI) (RPMI 1640, BioWhittaker, Walkersvile, MD) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Gaithersburg, MD), glutamine (2 mM, BioWhittaker), penicillin (100 U/ml, BioWhittaker), streptomycin (100 μg/ml, BioWhittaker), 1× nonessential amino acids (Sigma, St. Louis, MO), 2-ME (5×10^{-5} M, Sigma), and sodium pyruvate (1 mM, BioWhittaker). The 4T1-9 tumor, a clone of 4T1, was kindly provided by Dr. Bernard Fox, Portland, OR. The R1 and RA5 tumors were maintained in cRPMI containing 800 ug/ml G418 (BioWhittaker).

2.2. Generation of anti-sense CCL5 expressing tumors

Messenger RNA (mRNA) was isolated from 1×10^6 4T1-9 cells according to the Oligotex Direct mRNA Protocol (Qiagen, Valencia, CA). The mRNA was converted to cDNA using random primers and MMTV-RT (Promega Corporation, Madison, WI). RT-PCR was used to amplify the 202 bp fragment of CCL5 that was cloned into the pGEM T-easy vector (Promega). The CCL5 insert was cut out of the pGEM T-vector with EcoR1 and ligated into the eukaryotic expression vector pcDNA3.1 (Invitrogen). Subsequently, clones containing CCL5 in the sense and anti-sense orientation were identified by sequencing (Pennsylvania State University Core Facility). Twenty-four hours prior to transfection the 4T1-9 tumor cells were plated in a 24-well plate at a concentration of 1×10^4 cells/ml of cRPMI (without penicillin and streptomycin) and incubated in a humidified chamber at 37 °C, 5.0% CO_2. The eukaryotic expression vector was linearized by digestion with *pvu*I and purified using the Qiaex II gel extraction kit (Qiagen) according to manufacture's instructions. Next, 6 ul of the lipid transfection reagent FuGene 6 (Roche Molecular Biochemicals, Indianapolis, IN) was added to microcentrifuge tubes containing 94 ul of Opti-MEM (Gibco BRL). The solution was gently mixed while 1 μg of DNA was added. Following a 20 min incubation the cRPMI was removed from the 4T1-9 cells plated 24 h earlier, 0.5 ml of cRPMI (without penicillin and streptomycin) was added, and the FuGene 6/DNA mixture was added drop wise to the cells. The plate was incubated at 37 °C and 5.0% CO_2 for 24 h. After this incubation, the cRPMI was removed and the cells were trypsinized and transferred to T-25 culture flasks. After 3 weeks of selection in G418, the tumor cells were cloned at 0.5 cells/well and screened in order to identify tumors for further study. The tumors were screened for CCL5 gene and protein expression via RT-PCR and ELISA, respectively. All tumors expressed similar levels of MHC Class I and no detectable MHC Class II (University of Pennsylvania Cancer Center, Flow Cytometry Facility, data not shown).

2.3. RT-PCR and gene array

PCR was used to amplify the fragment of CCL5 used to generate the anti-sense expression vector. For the PCR reactions mRNA was isolated from 1×10^6 cells using the Qiagen mRNA isolation kit, and converted to cDNA using random primers and MMTV-RT as described above. Three microliter of dH_2O was added in place of the cDNA to the negative control tubes. The reactions proceeded for 25–30 cycles: 94 °C for 15 s, 59 °C for 30 s, 74 °C for 45 s, in an MJ Research Thermocycler (MJ Research, Waltham, MA). Resulting PCR products were analyzed on a 2% agarose gel using the Alpha Innotech Gel Documentation System (Alpha Innotech Corp., San Leandro, CA).

CCL5 (product size 202 bp)	Sense 5′ GTGCCAACCCA-GAGAAGAAGT 3′ Anti-sense 5′ AGCTGAGATGCCCATTTT CC 3′

For the gene array mRNA was isolated from 5×10^6 splenic T cells that were incubated with 0.1 ug/ml CCL5 (Peprotech, Rocky Hill, NJ) dissolved in 0.1% bovine serum albumin (BSA, Sigma) or 0.1% BSA as a control for 72 h. Messenger RNA was also isolated from 1×10^6 R1 and RA5 tumors cells. The mRNA was converted to biotin labeled cDNA probes and hybridized to the gene array (mouse G protein-coupled receptors signaling pathwayfinder gene array) according to manufacturer's instructions (Superray Bioscience Corp., Frederick, MD). The arrays were

developed by enhanced chemiluminescence and analyzed on a gel documentation system (Alpha Innotech Corp.). Following densitometry analysis, the background was normalized to housekeeping genes, and the optical density of the quadruplicate spots was analyzed.

2.4. ELISA

To quantify CCL5 production the tumor cells were plated at 1×10^6 cells/well in a 24-well culture plate. After 1, 4, 8, or 24 h the supernatants were harvested, centrifuged for 5 min at $350 \times g$, transferred to a new microcentrifuge tube, and stored at −20 °C. The Quantikine ELISA kit (R&D Systems, Minneapolis, MN) was used to determine the amount of CCL5 present in the cell supernatants. In order to quantify the levels of IFN-γ in supernatants from the cytokine release assay an IFN-γ specific ELISA was used (R&D Systems).

2.5. Growth kinetics

Growth rates of the tumor cells were determined in vitro and in vivo. The in vitro growth kinetics were performed by plating 1×10^4 tumor cells/flask in cRPMI. Separate flasks were set up for 24, 48, 72, 96, and 120 h time points in duplicate. The cells were harvested by trypsinization and counted using a hemacytometer and trypan blue exclusion at the designated time points.

The in vivo growth kinetics were performed by injecting 5×10^4 cells/mouse subcutaneously in the hind flank and monitoring tumor growth every 2–3 days. The tumor volume was calculated by measuring the length and width of the tumor using a caliper then using the formula $(lxw^2)/2$.

2.6. Analysis of tumor infiltrating cells

Tumors were removed, minced and digested in a collagenase cocktail (1 mg/ml collagenase type 4, 20 ug/ml DNase, 10 U/ml hyaluronidase, Worthington Biochemical Corp., Lakewood, NJ) for 2 h at room temperature. The differential cell counts were performed with a cytospin (Cytospin 3, Shandon Inc., Pittsburgh, PA) of 5×10^4 cells which were stained using the Hema 3 staining kit (Fisher Scientific, Pittsburgh, PA). Three separate fields of view/slide were evaluated. For flow cytometric analysis the lymphocytes were enriched by passage over a nylon wool column followed by plastic adherance. Next, following a 20 min Fc block (CD16, CD32), the cells were stained with CD3 (FITC), CD4 (PE), CD8 (PE), NK (PE) specific antibodies or isotype controls (BD Pharmingen, San Diego, CA) for 30 min on ice. The cells were washed, fixed in 2% paraformaldehyde and analyzed by the University of Pennsylvania Cancer Center, Flow Cytometry Facility.

2.7. Chemotaxis assay

Spleens from naive and tumor-bearing mice were removed and T cells were enriched by passage over a nylon wool column. The resulting cell population was 85–95% pure $CD3^+$ T cells. A 96 well chemotaxis chamber (Neuro Probe Inc., Gaithersburg, MD) was used for the chemotaxis assay as previously described [7]. Briefly, the bottom wells of the chamber were loaded with recombinant CCL21 (Peprotech) resuspended in HBSS +0.1% BSA. The upper wells of the chemotaxis chamber, containing 1×10^5 effector cells, was separated from the lower wells by a 5 μm polyvinylpyrrolidone-free polycarbonate filter with adhesive pre-coated with murine laminin (GibcoBRL). Following a 1 h incubation at 37 °C, the filter and plate were centrifuged at $400 \times g$ for 5 min. The cells that had migrated were removed from the lower wells and counted with a hemacytometer. Duplicate samples were counted two times and evaluated for statistical significance with a Student's *t*-test.

2.8. Draining lymph nodes

Tumor draining (inguinal) lymph nodes were harvested 8 days after a subcutaneous injection of 1×10^6 tumor cells in both hind flanks of eight mice with the R1 tumor and eight mice with the RA5 tumor. Lymphocytes were removed by pressing the lymph nodes with the flat end of a syringe plunger. Cells expressing low/negative levels of CD62L ($CD62L^{lo}$) were isolated by negative selection using CD62L microbeads and expanded as previously described [9]. To measure cytokine production, $CD62L^{lo}$ cells were collected and incubated (1×10^5 cells/well) with media as a negative control or tumor targets (1×10^4 cells/well) in a 96-well plate. Twenty-four hours later the supernatants were harvested, centrifuged for 5 min at $350 \times g$, transferred to a new microcentrifuge tube, and stored at −20 °C.

3. Results

3.1. Modulation of tumor-derived CCL5

Previously we reported that the 4T1 tumor cell line constitutively produces CCL5 [7]. To study the role of tumor-derived CCL5 we down-regulated production using an anti-sense eukaryotic expression vector. Examination of tumor supernatants revealed that the R1 tumors (vector transfected control) produced normal levels of CCL5, whereas, the RA5 tumors (anti-sense transfectants) produced approximately 10-fold less CCL5 (Fig. 1A). In 24 h 1×10^6 R1 tumors produced 500 pg/ml of CCL5, whereas the RA5 tumors produced 50 pg/ml. To determine the stability of CCL5 expression the tumors were passaged in mice. After 4 weeks of growth in vivo the tumors were isolated, digested in a collagenase cocktail and assessed for CCL5 production. The R1 tumors maintained normal levels of CCL5 expression and the RA5 tumors maintained low levels of CCL5 expression (Fig. 1B). In addition, CCL5 production was assessed after the tumors were grown in vitro, in the absence of drug selection, for 1 month and the

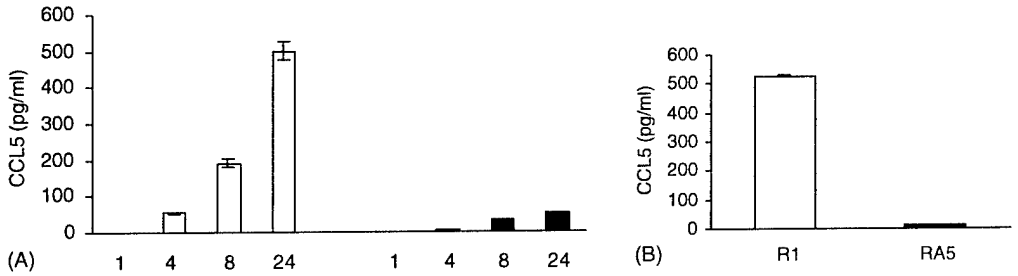

Fig. 1. Tumor-derived CCL5 production. (A) 4T1 tumors transfected with CCL5 in the anti-sense (■) orientation or vector transfected control (□) were screened for CCL5 production by ELISA. For this purpose 1×10^6 cells were plated in a 24-well plate and supernatants were harvested after 1–24 h in culture and assayed for CCL5. The data are representative of three separate experiments with standard deviation of duplicate wells shown. (B) The tumors were grown in vivo for 4 weeks, harvested and assayed for CCL5 production by placing 1×10^6 cells/well in a 24-well plate for 24 h. The experiment was repeated three times with similar results. The average and standard deviation of duplicate wells are shown.

expression pattern was maintained (data not shown). These data demonstrate the generation of 4T1 tumors that express different levels of CCL5.

3.2. Modulation of tumor growth

To determine whether tumor-derived CCL5 influenced the growth rate of the tumor we examined the in vitro and in vivo growth rates of the R1 and RA5 tumors. There was no difference in the growth rates between the R1 and RA5 tumors in vitro (Fig. 2A). However, the RA5 tumors grew at a slower rate than the R1 tumors in vivo (Fig. 2B). After 26 days of growth the average volume of the R1 tumors were 1188 mm^3, whereas, the average volume of the RA5 tumors were 319 mm^3. As a result, these data indicated that tumor-derived CCL5 modulated the in vivo, but not the in vitro growth rate of the tumors.

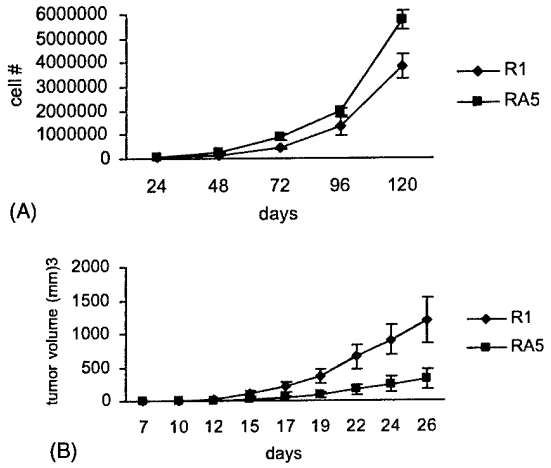

Fig. 2. Growth kinetics of the R1 and RA5 tumors. (A) In order to evaluate the in vitro growth rate of the CCL5 modified tumor cells equal numbers of R1 and RA5 tumors were plated on day zero and harvested for analysis after 24–120 h in culture. The data shown are representative of three separate experiments. (B) In order to evaluate the in vivo growth rate mice were injected with 5×10^4 R1 or RA5 and followed for tumor growth. The data are from one of three separate experiments with 5–10 mice/group. The average tumor volumes of 10 mice are shown.

3.3. Modulation of T cell response

Next, to establish whether modulation of a T cell response was associated with the difference in the in vivo growth rates of the tumors we examined 28-day tumors for the presence of tumor infiltrating lymphocytes. Analysis revealed that more lymphocytes and less macrophages were localized in the RA5 compared to the R1 tumors (Fig. 3A). Lymphocytes accounted for 56% of the white blood cells in the RA5 tumors and 15% of the white blood cells in the R1 tumors. Macrophages accounted for the majority of the remaining cells with few neutrophils found in either tumor. Similar observations were made after 2 weeks of tumor growth indicating that the increased number of T cells in the RA5 tumor persisted from as early as 2 weeks and as late as 4 weeks of tumor growth. Phenotypic analysis of the tumor infiltrates revealed an absence of natural killer cells and that the lymphocytes were all CD3+ T cells (Fig. 3B). The T cells infiltrating both the RA5 and R1 tumors were CD3/CD4 and CD3/CD8+ T cells present in approximately a 2:1 ratio CD4:CD8. Thus, down-regulation of tumor-derived CCL5 correlated with an increased number of tumor infiltrating lymphocytes.

Because we previously reported that T cells from mice bearing 4T1 tumors exhibited an impaired chemotactic ability [7] we investigated whether modulation of tumor-derived CCL5 influenced T cell chemotactic activity in tumor-bearing mice. For this purpose a chemotaxis assay was performed using splenocytes from naïve and tumor-bearing mice. We found that while mice bearing 4T1 and R1 tumors possessed splenic T cells that exhibited decreased chemotactic activity, mice bearing RA5 tumors possessed splenic T cells that migrated as well as splenic T cells from naïve mice (Fig. 4). Thus, inhibition of tumor-derived CCL5 prevented the tumor-associated alteration in chemotactic activity.

3.4. Examination of tumor draining lymph nodes

Because we could not isolate enough tumor infiltrating lymphocytes in high purity we examined $CD62L^{lo}$ cells

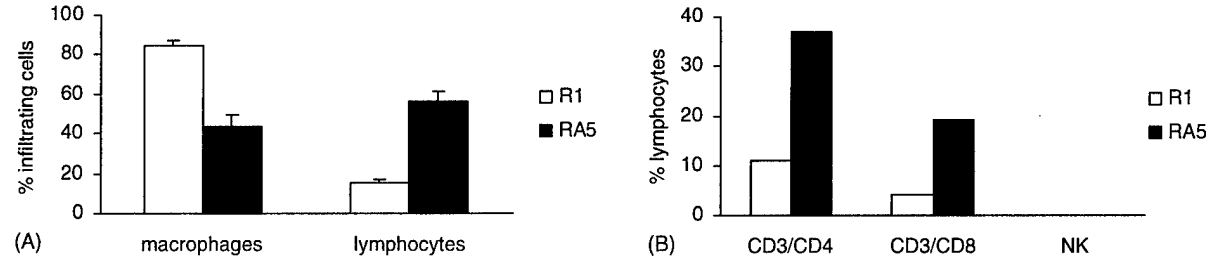

Fig. 3. Enhanced localization of T cells to RA5 tumors. (A) Balb/c mice were given R1 or RA5 tumors. Twenty-eight days later the tumors were harvested, digested in a collagenase cocktail and differential cell counts performed. The data represent one of seven separate tumors analyzed with the average and standard deviation of three fields of view shown. (B) Flow cytometry analysis was used to determine the percentage of CD3/CD4, CD3/CD8 and NK cells present in the lymphocyte populations. The data are representative of two separate experiments with 5–6 tumors/group.

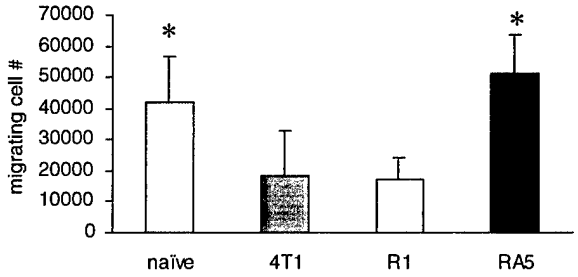

Fig. 4. Chemotactic activity of splenic T cells. Splenic T cells were isolated from mice bearing 4T1, R1, RA5 tumors and naïve mice. The splenic T cells were assayed for chemotactic activity toward CCL21 (SLC) in a 1 h chemotaxis assay. Data from three separate experiments were pulled with average and standard error shown. Where indicated (*) $P < 0.05$ migration was significantly different than in mice bearing parental 4T1 tumors.

from tumor-draining lymph nodes to determine whether the reduction in tumor-derived CCL5 correlated with an enhanced tumor-specific T cell response. Previously Kagamu et al. [10] reported that $CD62L^{lo}$ cells from tumor-draining lymph nodes contained the tumor-specific T cells. Here, we gave mice R1 or RA5 tumors and after 8 days harvested draining lymph nodes, isolated the $CD62L^{lo}$ cells and analyzed the specificity of these cells in a cytokine release assay (Fig. 5). T cells from lymph nodes draining RA5 tumors produced IFN-γ in response to the parental 4T1 tumor (674 pg/ml), but not a syngeneic murine mammary carcinoma SM1 (68 pg/ml). On the contrary, T cells from lymph nodes draining R1 tumors failed to make a tumor-specific IFN-γ response. As a result, these data indicated that inhibition of tumor-derived CCL5 enhanced the generation of a tumor-specific T cell response.

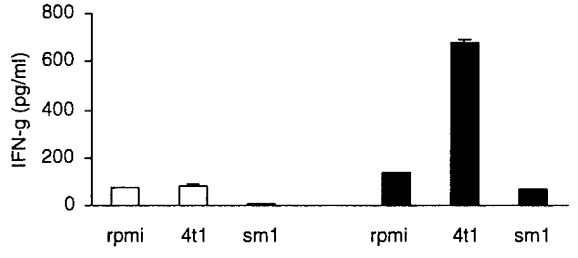

Fig. 5. Tumor-specific T cell response. In order to evaluate whether the R1 and RA5 tumors elicited different T cell responses the recently activated T cells ($CD62L^{lo}$) from the tumor draining lymph nodes were assessed in a cytokine release assay. The data are representative of three separate experiments with standard deviation of duplicate wells shown.

3.5. Tumor growth in immunodeficient mice

To validate the importance of CCL5 in modulating the T cell response to the tumor we examined growth in SCID mice. The data demonstrated that RA5 tumors grew faster in SCID mice than BALB/c mice supporting the contention that a T cell response in the BALB/c mice inhibited tumor growth (Fig. 6A). The RA5 tumors were 138 mm^3 after 17 days of growth in the SCID mice whereas they were 36 mm^3 in the BALB/c mice. Yet, the RA5 tumors grew slower than the R1 tumors in the SCID mice (Fig. 6B). The RA5 tumors were 138 mm^3 after 17 days of growth whereas the R1 tumors

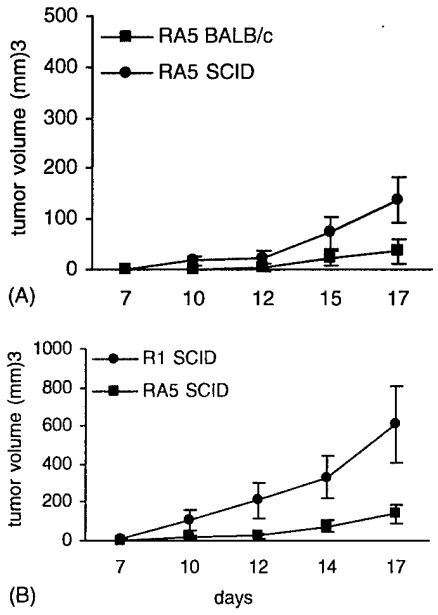

Fig. 6. Tumor growth in SCID mice. Mice were injected with 5×10^4 R1 or RA5 and followed for tumor growth. The data shown are representative of three separate experiments with 5–6 mice/group.

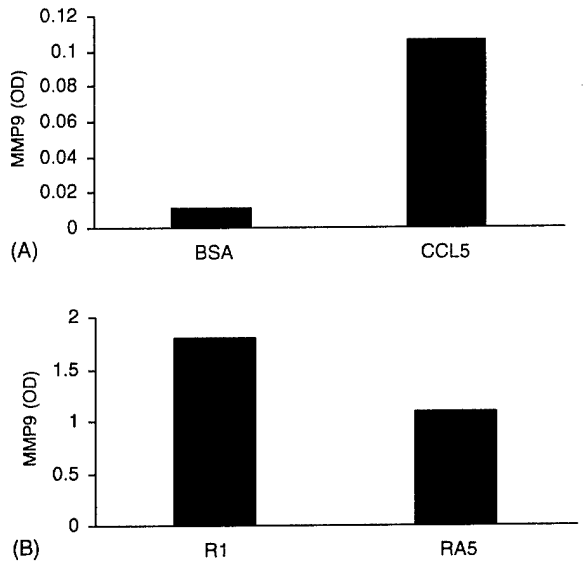

Fig. 7. CCL5 induced gene expression. (A) Splenic T cells were exposed to CCL5 or BSA as a control and gene expression was screened using a gene array. (B) Gene expression differences between R1 and RA5 were determined in a similar fashion. The level of MMP9 transcription was normalized to the ribosomal protein L13a housekeeping gene.

were 607 mm^3 after 17 days of growth. Consequently, the decreased in vivo growth rate of the tumor that expressed lower levels of CCL5 was not completely due to an enhanced T cell response.

3.6. CCL5 induced alteration in gene expression

Finally, in an attempt to identify a potential mechanism by which CCL5 could influence in vivo, but not in vitro tumor growth, we used a gene array. For this purpose we compared gene expression in T cells incubated with CCL5 or bovine serum albumin as a control (Fig. 7A). Among the genes regulated at the transcriptional level by CCL5, MMP9 was markedly upregulated and could account for an increased growth rate in vivo through enhancement of angiogenesis [8]. Since the tumors expressed the CCL5 receptors (data not shown), we looked at whether tumor-derived CCL5 influenced MMP9 transcription in an autocrine fashion. We found that down-regulation of tumor-derived CCL5 correlated with lower levels of MMP9 expression (Fig. 7B). As a result, these data suggested that tumor-derived CCL5 could enhance in vivo growth by inducing transcription of MMP9, which can contribute to angiogenesis. Additional studies are currently underway to further explore these findings.

4. Discussion

Only a few studies have described the role of CCL5 in murine tumor models. Mulé et al. [11] reported that transduction of the fibrosarcoma WP4 (a clone of MCA-205) with the CCL5 gene reduced tumorigenicity, and left animals with long term protection from parental tumor challenge. Depletion of $CD8^+$ T cells confirmed their role in tumor immunity. CCL5 expression was also beneficial in the EL4 murine lymphoma model [12]. Kutubuddin et al. [12] reported that direct injection of HSVB7.1 and HSVCCL5 into established EL-4 tumors, led to complete tumor regression and the generation of tumor-specific CTL activity. These data indicated that CCL5 was capable of generating tumor-specific CTL and was effective in generating anti-tumor immunity in a murine fibrosarcoma and lymphoma model. Yet, there is a paucity of information about the role of CCL5 in anti-tumor immunity in murine mammary carcinoma models. However, it has been reported that murine mammary carcinomas and specimens from patients with breast cancer constitutively express this chemokine.

Two studies have investigated the expression of CCL5 by clinical specimens. Niwa et al. [1] examined 43 breast cancer specimens for CCL5 expression and reported that plasma levels of CCL5 were greater in patients with progressive disease compared to those in clinical remission [1]. Moreover, plasma levels correlated with disease stage with more advanced stages correlating with higher levels of CCL5 expression [1]. In the other study Luboshits et al. [2] examined sections from breast cancer specimens and reported that 74% of the sections exhibited CCL5 expression, whereas normal epithelial cells, ductal epithelial cells nor benign sections showed similar levels of CCL5 expression. Azenshtein et al. [3] reported the expression of MMP9 may be regulated by CCL5 and as a result CCL5 may play a role in invasion and metastases. Thus, given that breast cancer specimens often constitutively produce CCL5 it would appear that production of this chemokine is not beneficial to the generation of an anti-tumor immune response. Due to the frequency of expression and lack of information about its role in tumor immunity we wanted to address the role of tumor-derived CCL5 in a murine mammary carcinoma model.

Previously, we reported that the murine mammary carcinoma 4T1 and spontaneous breast cancers from rat neu transgenic mice constitutively express CCL5 [7,13]. For this study we pursued the role of CCL5 in the 4T1 cell line. In order to begin addressing the role of this chemokine in anti-tumor immunity we down-regulated CCL5 expression using an anti-sense eukaryotic expression vector. A vector transfected control that expressed the same levels of CCL5 as the parental tumor was used for comparative purposes. Using these cells were able to address whether, and the extent to which, tumor-derived CCL5 impacted the T cell response to 4T1.

The data reported here revealed that CCL5 produced by 4T1 has multiple roles. First, we found that tumors, which expressed lower levels of CCL5, elicited a better T cell response. For instance, there was a greater T cell infiltration into the tumors that express lower levels of CCL5. Although, this may seem paradoxal these data make sense in light of the chemotaxis data. Previously we reported that mice bearing

4T1 tumors possessed splenic T cells with abnormal chemotactic activity [7]. The idea was that as a tumor, that constitutively expresses chemokines, grows the chemokine receptors in peripheral T cells would become desensitized because of persistent activation and consequently, lose chemotactic activity. We report here that inhibition of tumor-derived CCL5 prevented the altered chemotactic activity and therefore, one would expect a greater number of T cells to make it to the tumor site. In addition, inhibition of tumor-derived CCL5 also enhanced the tumor-specific T cell response evident in the tumor draining lymph nodes. This may be due to a direct effect of persistent exposure of the T cells to CCL5 because we have found that prolonged incubation of T cells with recombinant CCL5 impairs TCR capping capability and the ability of the T cells to produce IFN-γ upon CD3 crosslinking (data not shown). Consequently, tumor-derived CCL5 may impair the ability of the T cells to produce IFN-γ by impairing the ability to cap the TCR. These studies are ongoing. Another possible explanation for how tumor-derived CCL5 could impair the T cell response relates to the ability of CCL5 to recruit dendritic cells. In particular, CCL5 has been reported to recruit immature dendritic cells [14,15]. Consequently, it is possible that the tumor-derived CCL5 recruits immature DC which then tolerize the T cells. If this occurs one would expect to find a greater proportion of immature DC infiltrating the R1 versus RA5 tumors. In addition, these DC would be selectively capable of tolerizing rather than activating a T cell response. These studies are also ongoing.

The second major role of tumor-derived CCL5 was revealed in SCID mice. The difference in growth rates of the R1 and RA5 tumors in SCID mice suggested that tumor-derived CCL5 also has the capacity to modulate tumor growth in vivo. The ability of CCL5 to regulate transcription of MMP9 could at least be partially responsible for these findings.

Regardless of the method by which tumor-derived CCL5 impairs the T cell response and/or enhances the growth of breast cancer, there are a number of potential methods to counter these effects. Because HIV utilizes the CCL5 receptor (CCR5) for cellular entry [16], there has been an immense amount of research into the practicality of blocking the activity of this receptor and in the generation of receptor antagonists. For instance, natural products such as cyclophilin from *Toxoplasma gondii* to synthetic products are being evaluated for their ability to bind and inhibit CCR5 [17,18]. Also, because CCL5 has been implicated in adverse inflammatory responses associated with diseases such as arthritis, asthma and granuloma formation [19–21], inhibitors of CCR5 are being developed and evaluated for treatment of those diseases as well [22]. As a result, it would be interesting to explore whether such inhibitors could be beneficial in a tumor setting at either preventing tumor-derived CCL5 from impairing the T cell response or preventing it from enhancing growth of the tumor in vivo. At the same time such inhibitors could prove useful in determining whether there is a specific level of inhibition that is most beneficial to increase the T cell response and/or decrease tumor growth.

Collectively, the data here indicate that CCL5 produced by 4T1 is capable of inhibiting T cell mediated anti-tumor immunity and enhancing tumor growth in vivo. Although, many questions remain unanswered, and this study has undoubtedly raised several new questions, one important aspect is clear; i.e. the role of tumor-derived CCL5 in breast cancer warrants more attention than that obtained thus far.

Acknowledgements

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Impact of tumor-derived CCL2 on T cell effector function

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Abstract

To study the effects of tumor-derived monocyte chemoattractant protein-1 (MCP-1, CCL2) on the anti-tumor immune response we used the 4T1 murine mammary carcinoma which constitutively expresses CCL2. We generated 4T1 that do not express detectable levels of CCL2 and found that the T cell response to the tumors were altered. Lymph nodes draining the CCL2- tumor contained $CD62L^{lo}$ cells that produced greater levels of INF-γ in response to the tumor than $CD62L^{lo}$ cells from lymph nodes draining a tumor that produced CCL2. Moreover, exposure of splenic T cells to recombinant CCL2 in vitro decreased the ability of the T cells to produce IFN-γ. However, despite the enhanced effector function evident in the absence of CCL2, vaccination/challenge experiments failed to reveal an increase in immunogencity of the CCL2 null cells relative to the $CCL2^{+}$ cells. Collectively, these data indicate that tumor-derived CCL2 could decrease T cell effector function, yet not the overall immunogenicity of the tumor.

Keywords: Tumor Immunity; 4T1; CCL2; MCP-1

1. Introduction

In 1983 Botazzi et al. [1] reported that the amount of tumor derived chemotactic factor (TDCF) produced by tumors correlated with the level of macrophage infiltration. Subsequently, monocyte chemoattractant protein-1 (MCP-1, CCL2) was found to be responsible for most of the biological effects of TDCF [2]. CCL2 is a CC chemokine that is capable of acting upon macrophages as well as T cells and basophils [3]. Following these initial studies multiple reports documented that additional human and murine tumors constitutively express CCL2 [4–6]. Yet the role of tumor-derived CCL2 in tumorigenicity and anti-tumor immunity has not been resolved. In fact, the field has been complicated by contrasting results.

Several reports have indicated that tumor-derived CCL2 can enhance progression of breast cancer in humans and increase malignancy of murine mammary tumors [5,6]. The ability of CCL2 to recruit macrophages may be one mechanism by which CCL2 can mediate some of these effects [7]. In contrast, several studies have reported that introduction of the CCL2 gene into tumor cells correlated with decreased tumorigenicity and facilitated immune mediated tumor rejection [8–10]. Thus, the precise role of CCL2 in anti-tumor immunity remains unsettled. While many investigators have examined the influence of tumor-derived CCL2 on macrophages, the impact of CCL2 on the T cell response to cancer has for the most part been overlooked. One study that did focus on T cells reported that MCA205-derived CCL2 impaired T cell effector function [11].

In this study we were interested in determining how tumor-derived CCL2 influenced the T cell response to the 4T1 murine mammary carcinoma model. 4T1 is a relatively weakly immunogenic tumor cell line that constitutively expresses CCL2 [12]. Previously we reported that mice bearing the 4T1 tumor possess splenic T cells with altered chemokine receptor function [12]. Here we wanted to know whether inhibiting the tumor cells from producing CCL2 would influence the T cell response and consequently the immunogenicity of the tumor. For this reason we generated cells that lacked detectable levels messenger RNA encoding CCL2 and CCL2 protein. Next, we compared the T cell response elicited by the CCL2 null cells to the response elicited by the cells that produced CCL2. Analysis revealed that blockade of tumor-derived CCL2 could increase T cell effector function, but not the immunogenicity of the tumor.

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2. Materials and methods

2.1. Mice and tumor cell lines

For these studies 6–8 week old female BALB/c mice were used. The BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Food and water were provided ad libitum. All of the tumor cells used in this study (4T1, 4T1-9, 4T1-A4, 4T1-G7, and SM1) were maintained in complete RPMI (cRPMI) (RPMI 1640, BioWhittaker, Walkersvile, MD) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Gaithersburg, MD), glutamine (2 mM, BioWhittaker), penicillin (100 U/mL, BioWhittaker), streptomycin (100 μg/ml, BioWhittaker), 1X nonessential amino acids (Sigma, St. Lois, MO), 2-ME (5×10^{-5} M, Sigma), and sodium pyruvate (1 mM, BioWhittaker). The 4T1-9 tumor, a clone of 4T1, was kindly provided by Dr. Bernard Fox, Portland, OR. The 4T1-A4 and 4T1-G7 clones were maintained in cRPMI containing 800 ug/ml G418 (BioWhittaker).

2.2. Generation of CCL2+ and CCL2- cells

Messenger RNA (mRNA) was isolated from 1×10^6 4T1-9 cells according to the Oligotex Direct mRNA Protocol (Qiagen, Valencia, CA). The mRNA was converted to cDNA using random primers and MMTV-RT (Promega Corporation, Madison, WI). RT-PCR was used to amplify a 345 bp fragment of CCL2 that was cloned into the pGEM T-easy vector (Promega). The CCL2 insert was cut out of the pGEM T-vector with EcoR1 and ligated into the eukaryotic expression vector pcDNA3.1 (Invitrogen). Subsequently, vectors containing CCL2 in the sense and anti-sense orientation were identified by sequencing (Pennsylvania State University Core Facility). Twenty-four hours prior to transfection the 4T1-9 tumor cells were cultured in a 24-well plate at a concentration of 1×10^4 cells/ml of cRPMI (without penicillin and streptomycin) and incubated in a humidified chamber at 37 °C, 5.0% CO_2. Both sense and anti-sense eukaryotic expression vectors were linearized by digestion with *pvu*I and purified using the Qiaex II gel extraction kit (Qiagen) according to manufacture's instructions. Next, 6 ul of the lipid transfection reagent FuGene 6 (Roche Molecular Biochemicals, Indianapolis, IN) was added to microcentrifuge tubes containing 94 ul of Opti-MEM (Gibco BRL). The solution was gently mixed while 1 μg of the sense or antisense DNA was added. Following a 20 min incubation, the cRPMI was removed from the 4T1-9 cells plated 24 h earlier, 0.5 ml of cRPMI (without penicillin and streptomycin) was added, and the FuGene 6/DNA mixture was added drop wise to the cells. The plate was incubated at 37 °C and 5.0% CO_2 for 24 h. After this incubation, the cRPMI was removed and the cells were trypsinized and transferred to T-25 culture flasks. After three weeks of selection in G418, the tumor cells were cloned at 0.5 cells/well and screened in order to identify sense and anti-sense cells for further study. The cells were screened for CCL2 gene and protein expression via RT-PCR and ELISA respectively. All cells expressed similar levels of MHC Class I and no detectable MHC Class II (University of Pennsylvania Cancer Center, Flow Cytometry Facility, data not shown).

2.3. RT-PCR

For all PCR reactions mRNA was isolated from 1×10^6 cells using the Qiagen mRNA isolation kit, and converted to cDNA using random primers and MMTV-RT as described above. Three μl of dH_2O was added in place of the cDNA to the negative control tubes. The reactions proceeded for 25–30 cycles: 94 °C for 15 s, 59 °C for 30 s, 74 °C for 45 s, in an MJ Research Thermocycler (MJ Research, Waltham, MA). Resulting PCR products were analyzed on a 2% agarose gel using the Alpha Innotech Gel Documentation System (Alpha Innotech Corp., San Leandro, CA).

CCL2 (product size 345 bp)	sense 5′ CAC TCA CCT GCT GCT ACT CAT T 3′ antisense 5′ TCA CAC TGG TCA CTC CTA CAG AA 3′
GAPDH (product size 212 bp)	sense 5′ CAGGTTGTCTCCTGC-GACTT 3′ antisense 5′ CTTGCTCAGTGTC-CTTGCTG 3′

The CCL2 PCR product was the same one used to generate the sense and anti-sense expression vectors. The Vβ TCR primers have been previously described [13]. Densitometry was performed using the analysis software of the Alpha Innotech System.

2.4. ELISA

To quantify CCL2 production the tumor cells were plated at 1×10^6 cells/well in a 24-well culture plate. After 1, 4, 8, or 24 h the supernatants were harvested, centrifuged for 5 min at $350 \times g$, transferred to a new microcentrifuge tube, and stored at −20 °C. The Quantikine ELISA kit (R&D Systems, Minneapolis, MN) was used to determine the amount of CCL2 present in the cell supernatants. In order to quantify the levels of IFN-γ and IL-4 in supernatants from the cytokine release assay IFN-γ and IL-4 specific ELISAs were used (R&D Systems).

2.5. Growth kinetics

Growth rates of the tumor cells were determined in vitro and in vivo. The in vitro growth kinetics were performed by plating 1×10^4 tumor cells/flask in cRPMI. Separate flasks were set up for 24, 48, 72, 96, and 120 h time points in duplicate. The cells were harvested by trypsinization and counted using a hemocytometer and trypan blue exclusion at the designated time points.

The in vivo growth kinetics were performed by injecting 5×10^4 cells/mouse subcutaneously in the hind flank and monitoring tumor growth every 2–3 days for 21 days. The tumor volume was calculated by measuring the length and width of the tumor using a caliper then using the formula $(lxw^2)/2$. For the vaccination/challenge experiments the mice were vaccinated in both hind flanks with 1×10^6 Mitomycin C treated (Roche, 50 ug/ml for 1 h at 37 °C) tumor cells 14 days prior to challenge with 5×10^4 tumor cells in the left flank.

2.6. Draining lymph nodes

Tumor draining (inguinal) lymph nodes were harvested eight days after a sc injection of 1×10^6 tumor cells in both hind flanks of eight mice with the A4 tumor and eight mice with the G7 tumor. Lymphocytes were removed by pressing the lymph nodes with the flat end of a syringe plunger. Cells expressing low/negative levels of CD62L ($CD62L^{lo}$) were isolated by negative selection using CD62L microbeads and expanded as previously described [14]. To measure cytokine production, $CD62L^{lo}$ cells were collected and incubated (1×10^5 cells/well) with media as a negative control or tumor targets (1×10^4 cells/well) in a 96-well plate. Twenty-four hours later the supernatants were harvested, centrifuged for 5 min at $350 \times g$, transferred to a new microcentrifuge tube, and stored at −20 °C.

3. Results

3.1. Characterization of CCL2- and CCL2+ tumor cells

In order to investigate the role of tumor-derived CCL2 in the T cell response to cancer we compared CCL2- and CCL2+ tumors. For this purpose we used cells derived from a clone of 4T1. The A4 cells expressed similar levels of mRNA for CCL2 compared to the parental tumor, whereas no message was detected from the CCL2- tumor; G7 (Fig. 1A). Analysis of supernatants, collected from the tumor cells, for CCL2 protein revealed a similar pattern. A4 expressed high levels of CCL2 whereas G7 produced no detectable CCL2 (sensitivity level 15 pg/ml, Fig. 1B). In order to evaluate the stability of CCL2 expression supernatants were assayed following in vivo passage of the tumors. Following 3 weeks of tumor growth the expression of CCL2 by G7 remained below detection and A4 maintained CCL2 expression (Fig. 1B). For this analysis, tumors were pulled from three mice bearing A4 or G7 tumors and analyzed for CCL2 expression. This process was repeated three times and therefore tumors from nine mice were analyzed in all. Additionally, the pattern of CCL2 expression was not altered following maintenance of the cells in vitro, in the absence of drug selection, for one month (data not shown). These results confirmed that A4 expressed CCL2, G7 did not, and that the expression was stable.

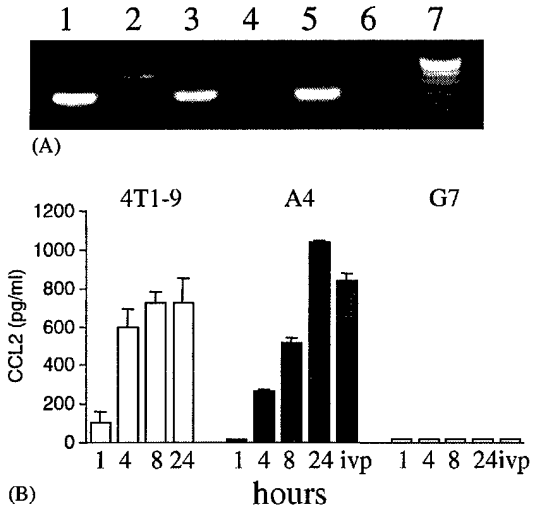

Fig. 1. Expression of CCL2. (A) Messenger RNA was isolated from 4T1-9 (lanes 1, 2), A4 (lanes 3, 4), and G7 (lanes 5, 6), converted to cDNA and analyzed for CCL2 (lanes 2, 4, 6) and GAPDH (lanes 1, 3, 5) by RT-PCR. Lane 7 contains the molecular weight marker. The results are representative of three separate experiments; (B) supernatants were harvested from 4T1-9 (□), A4 (■) and G7 (■) after 1, 4, 8, or 24 h in culture and assayed for CCL2 by ELISA. Twenty-four hour supernatants were also harvested from A4 and G7 after growth of the tumors for 3 weeks in vivo (in vivo passaged tumors, ivp). The error bars represent the standard deviation of duplicate wells analyzed by ELISA. The data are representative of three separate experiments.

3.2. Examination of in vitro and in vivo growth rates

As an initial comparison of the CCL2- and CCL2+ cells the in vitro and in vivo growth rates were compared to that of the parental tumor. The data revealed that the CCL2- and CCL2+ tumors grew at similar rates and were comparable to the parental tumor in vitro and in vivo (Fig. 2). These data indicated that the absence of CCL2 did not modulate the growth rate of the tumors, and thus these cells could prove useful in examining the impact of tumor-derived CCL2 on the effector T cell response.

3.3. Examination of tumor draining lymph nodes

In order to determine whether CCL2 modulation influenced the anti-tumor immune response we examined T cells from the tumor-draining lymph nodes in a cytokine release assay. In particular we examined T cells expressing low levels of CD62L ($CD62L^{lo}$) which has previously been reported to contain the tumor reactive T cell population [15]. The $CD62L^{lo}$ cells from lymph nodes draining the G7 tumors produced more IFN-γ in the cytokine release assay, than the $CD62L^{lo}$ cells from the lymph nodes draining the A4 tumor (Fig. 3A). These results indicated that the presence of CCL2 decreased the ability of the T cells to produce IFN-γ in reponse to the tumor. In order to determine whether a shift toward a type-2 cytokine response occured we also examined IL-4 levels. The data show that T cells from lymph

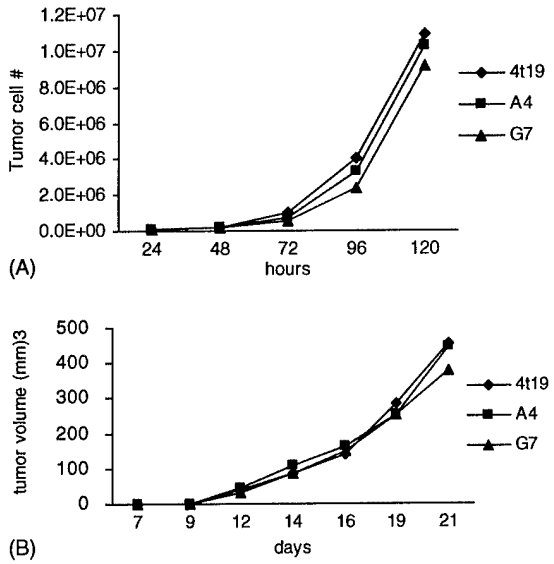

Fig. 2. Growth kinetics of CCL2 modified tumors. (A) In order to evaluate the in vitro growth kinetics of the CCL2 modified tumor cells equal numbers of A4 and G7 were plated on day 0 and harvested for analysis after 24–120 h in culture. The data shown are representative of three separate experiments; (B) in order to evaluate the in vivo growth kinetics mice were injected with 5×10^4 A4 or G7 and followed for tumor growth. The data are from one of three separate experiments with 4–5 mice per group. 4T1-9 was included for comparison purposes.

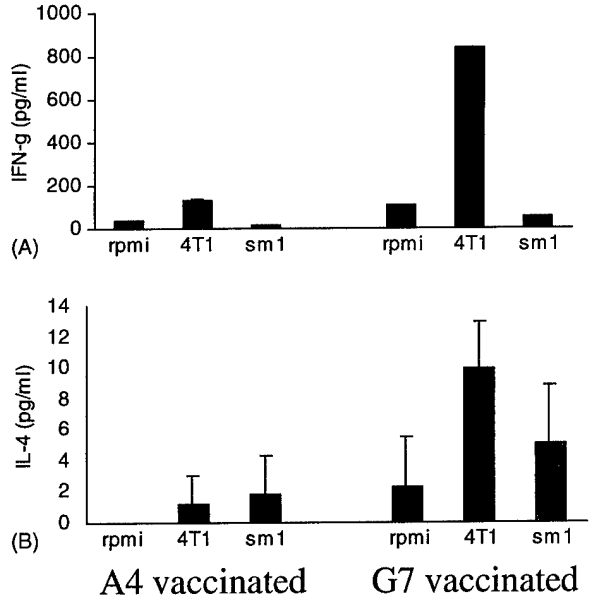

Fig. 3. Cytokine release assay. In order to evaluate the reactivity and specificity of the CD62L^{lo} cells from the tumor draining lymph nodes a cytokine release assay was used. CD62L^{lo} cells draining the A4 and G7 tumors were exposed to RPMI alone or tumor cells for 24 h and the supernatants were harvested and examined for IFN-γ (A) and IL-4 (B). The data are representative of three separate experiments with the standard deviation of duplicate wells of the ELISA shown. The SM1 is a syngeneic murine breast cancer cell line used as a specificity control.

nodes draining the G7 tumors produced more IL-4 than the T cells from lymph nodes draining the A4 tumors (Fig. 3B). Yet, the amount of tumor-specific IL-4 (10 pg/ml) was low relative to the levels of IFN-γ (836 pg/ml). These data indicated that tumor-derived CCL2 decreased the ability of the T cells to produce IFN-γ and did not induce a type-1 to type-2 cytokine switch.

In an attempt to determine whether there were any major differences in the population of T cells responding to the CCL2- and CCL2+ tumors we examined the Vβ TCR usage of the CD62L^{lo} T cell population from the tumor draining lymph nodes. The data revealed that the T cell subsets were similar. T cells bearing the TCR Vβ 1, 6, 8, 9, 10, and 13 predominated the response to the CCL2- and CCL2+ tumors (Fig. 4). These data indicated that the increased tumor-reactivity from the G7 tumor draining lymph nodes was not due to expansion of a particular T cell subset. However, it remains to be seen as to whether there are differences within particular Vβ families, which would become evident upon cloning the T cells or sequencing the CDR3 regions of the TCR.

3.4. Exposure of T cells to CCL2 in vitro

In order to determine whether CCL2 could directly modulate the ability of T cells to produce IFN-γ we exposed splenic T cells from naïve mice to recombinant CCL2 in vitro. For this purpose we used nylon wool enriched T cells (90% pure CD3$^+$ T cells, data not shown). We report that exposure of the T cells to varying doses of CCL2 modulated the ability of the T cells to produce IFN-γ (Fig. 5A). At the lowest dose of CCL2 examined (0.001 ug/ml) the T cells produced 250 ng/ml of IFN-γ whereas at 0.1 ug/ml of CCL2 the T cells produced 100 ng/ml of IFN-γ. This decrease in

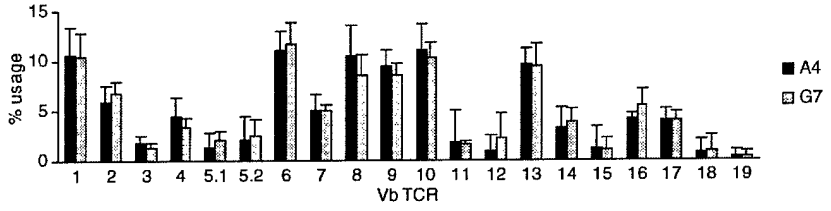

Fig. 4. T cell subset analysis in the tumor-draining lymph nodes. The CD62L^{lo} cells isolated from the tumor draining lymph nodes were examined for Vβ TCR usage by RT-PCR. Densitometric analysis was performed by comparing the density of the Vβ TCR PCR products to the GAPDH control. The data represent the average of three separate experiments with the standard deviation shown.

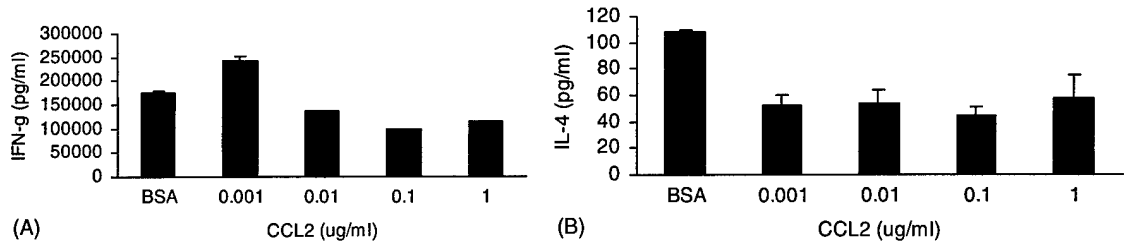

Fig. 5. Exposure of T cells to recombinant CCL2. To determine whether CCL2 exerted a direct influence on splenic T cells they were exposed to varying doses of recombinant CCL2 or bovine serum albumin (BSA) for 72 h. Subsequently, the cells were exposed to antibody to CD3 for 24 h and the supernatants were harvested and assayed for IFN-γ (A) and IL-4 (B). The results are representative of three separate experiments with the standard deviation of duplicate wells of the ELISA shown.

IFN-γ production was not attributed to a decrease in T cell viability nor an increase in T cell death. Viability of the T cells following exposure to the chemokine and the control cells remained similar (data not shown). The ability of the T cells to produce IL-4 was similarly not affected by CCL2 exposure (Fig. 5B). Thus, CCL2 directly influenced the ability of the T cells to produce IFN-γ. Additionally, similar to the data from the tumor-derived CCL2, recombinant CCL2 did not induce a type-1 to type-2 cytokine switch.

3.5. Vaccination/challenge experiments

Finally, we assayed the immunogenicity of the CCL2- and CCL2+ tumors using vaccination/challenge experiments. Due to the greater levels of IFN-γ produced by the T cells in response to the CCL2- cells we anticipated that the G7 tumors would be more immunogenic than the A4 tumors. In order to test this we immunized mice with Mitomycin C treated tumor cells and challenged the mice with A4 or G7. We found that the G7 tumor did not exhibit enhanced immunogenicity relative to the A4 tumor (Fig. 6). These data indicated that, although the G7 tumor elicited a greater T cell IFN-γ response, tumor immunogenicity was not increased by blockade of tumor-derived CCL2.

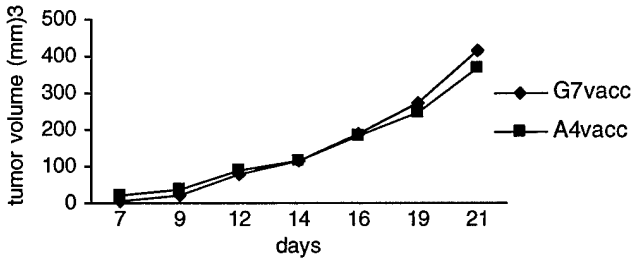

Fig. 6. Immunogencity of the A4 and G7 tumors. To evaluate the immunogenicity of the tumor cells vaccination/challenge experiments were used. Mice were vaccinated with 1×10^6 Mitomycin C treated A4 (A4vacc) or G7 (G7vacc) tumor cells and 14 days later challenged with 5×10^4 A4 or G7 and monitored for tumor growth. The data shown are the average of five separate animals from one experiment. The results are representative of three separate experiments with 5–8 mice per experiment.

4. Discussion

Due to contrasting results in different animal models the role of CCL2 in anti-tumor immunity has been difficult to unravel. Some investigators have reported that tumor-derived CCL2 decreased tumorigenicity. For instance, Chinese Hamster Ovary (CHO) cells transfected with either the human or murine CCL2 gene lose their ability to form tumors in nude mice [8]. Similarly, a delay in tumor growth occurred when a B16 derived cell line, B78HI, was transfected with the CCL2 gene [16]. However, in that study the results differed depending upon the dose of tumor cells delivered to the mice. When a lower number of tumor cells were delivered an increase in the tumorigenicity of the CCL2 modified cells was evident. The CT26 colon carcinoma and RENCA cell lines were used to evaluate whether CCL2 expression could affect metastasis. Huang et al. [9,17] reported that CCL2 decreased the metastatic potential as well as the tumorigenicity of these cell lines. Macrophages were associated with the ability of CCL2 to mediate these effects. NK cell recruitment has also been linked to the effects of CCL2. When CCL2 was introduced into the human lung adenocarcinoma cell line PC-14, NK cells were implicated in suppressing the systemic spread of disease [10]. Despite these findings, introduction of CCL2 into a clone of the CT26 cell line, C20, resulted in enhancement of lung metastasis [18]. The C20 cells are not unique in this regard, as other studies have reported that CCL2 expressing tumors exhibit increased tumorigenicity.

A comparison of two related murine mammary adenocarcinoma cell lines revealed a direct correlation between tumorigenicity and CCL2 expression [6]. In that study Neumark et al., [6] compared two cell lines that differed in malignant phenotype. The Ly-6hi DA3 cell line was more malignant and expressed higher levels of CCL2 whereas the Ly-6lo DA3 cell line expressed lower levels of CCL2 and were less malignant. Because macrophage infiltration has been correlated with the level of tumor-derived CCL2 several investigators have hypothesized that macrophages may play a role in the enhanced tumorigenicity and metastatic potential of CCL2 expressing tumors. This is related to the fact that CCL2 can elicit the release of enzymes from macrophages

that are capable of digesting the extracellular matrix and thus facilitate metastasis [7]. Taking a different approach toward investigating the relationship between CCL2 and macrophages, Asano et al., [19] inserted the CCL2 gene or anti-sense transcript into two human brain tumor cell lines; HBT28, which constitutively expressed high levels of CCL2, and HBT20, which expressed lower levels of CCL2. For that study CCL2 was down regulated in HBT28 and over-expressed in HBT20. The results were uninformative however because monocyte-mediated cytotoxicity against the HBT20-CCL2 transduced clone was increased, and the same effect was observed with the HBT28-anti-sense CCL2 transduced clone. One possible explanation for the contrasting results from many of these studies may be that the role of CCL2 is dependent upon the tumor type and the surrounding tumor environment.

Despite the differing functions attributed to CCL2 in various animal models the importance of delineating the role of CCL2 remains because a number of human tumors express this chemokine. Moreover, CCL2 expression has been reported as an indicator of relapse in breast cancer patients [5]. And, Salcedo et al., [20] reported that CCL2 could induce blood vessel formation and mice bearing human breast carcinoma cells survive longer, and had decreased lung metastasis, if the mice were treated with CCL2 neutralizing antibody. One common finding from the previous studies is that the impact of tumor-derived CCL2 on the T cell response to cancer has not received much attention.

In this study we focused on the impact of tumor-derived CCL2 on the T cell response to the 4T1 murine mammary carcinoma. We found that T cells from mice bearing CCL2 null tumors elicited a greater IFN-γ response to the tumors compared to T cells from mice bearing tumors that express CCL2. We also report that exposure of T cells to recombinant CCL2 in vitro decreased the ability of the T cells to produce IFN-γ upon stimulation with anti-CD3. Therefore, CCL2 directly modulated the ability of the T cells to produce IFN-γ. These data support the findings reported by another group. Peng et al., [11] investigated the role of CCL2 produced by the MCA205 sarcoma and reported that neutralization of this chemokine, while expanding tumor-specific T cells, improved the therapeutic efficacy of the T cells. Also, the T cells produced greater levels of IFN-γ in a cytokine release assay compared to T cells expanded in the presence of CCL2. Whether additional effector functions are similarly influenced by CCL2 is under investigation. One additional effector mechanism that warrants investigation is the cytotoxic T lymphocyte (CTL) activity and mechanisms by which it can be carried out such as FasL, TRAIL and perforin expression [21–23]. Unfortunately, the impact of tumor-derived CCL2 on cytolytic activity is more difficult to discern because tumor-specific T cells from vaccine draining lymph nodes are not cytolytic and CTL activity cannot be detected from splenic T cells from 4T1 tumor-bearing mice or freshly isolated tumor-infiltrating lymphocytes (TIL).

Although CCL2 has been reported to aid in the generation of type-2 cytokine responses [24] we did not find that the decrease in IFN-γ production was compensated with an increase in type-2 cytokine production. In this study the T cells produced greater levels of IFN-γ compared to IL-4 regardless of the presence or absence of CCL2. Therefore, the type-1 cytokine reponse elicited by the 4T1 tumor was not attributed to CCL2. In fact, there was slightly more IL-4 produced by the T cells responding to the CCL2 null tumors compared to the T cells responding to the CCL2 expressing tumors. However, despite the greater levels of IFN-γ produced in reponse to the tumors that lack CCL2 production, the immunogenicity of the tumors, when defined using vaccination/challenge experiments, was not enhanced.

One additional area to consider is the role of tumor-derived CCL2 in the altered chemokine receptor function we previously reported in tumor-bearing mice [12]. We examined the chemotactic activity of splenic T cells from tumor bearing mice and found altered chemokine receptor function in mice bearing the A4 and G7 cells (data not shown). Therefore, the expression of this chemokine by the tumor cells did not appear to be responsible for the altered chemokine receptor activity we previously reported.

Finally, although the influence of tumor-derived CCL2 on T cell effector function requires additional studies, the stable CCL2 null tumor cells can be used for additional studies. For instance, the role of tumor-derived CCL2 in the initial stages of the innate anti-tumor immune response can be examined. We are particularly interested in determining whether tumor-derived CCL2 plays a part in the early recruitment of macrophages to a growing tumor, and because 4T1 is capable of metastases the CCL2+ and CCL2- tumors may prove useful in examining the role of tumor-derived CCL2 in the metastatic potential of 4T1.

Acknowledgements

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